analogous to ARNT have been identified for PER. However, it has recently been shown that the PAS domain of the PER protein can function as a protein interaction domain (*14*). Further mapping of the *tim*-sensitive region of PER should determine whether PAS is centrally involved in the control of PER nuclear localization.

Peak amounts of *per* RNA expression (*11*, *12*) precede the greatest amount of PER protein staining in nuclei of the photoreceptors and brain (*8*) by about 8 hours. Thus, nuclear immunoreactivity of PER occurs when the amount of *per* RNA is small. As suggested above, the entry of PER protein into the nucleus may be under temporal control, so that some of the previously observed oscillation in PER protein amounts and nuclear staining (*8*, *10*) may reflect rhythmic movement between the cytoplasm and the nucleus, with a phase that is distinct from that of *per* RNA synthesis. In light of these observations and our work with *tim*, we speculate that formation of an intracellular circadian clock may require nuclear localization of PER to be limited to a particular time of day.

In such a model, constitutively cytoplasmic (as in *tim* mutants) or constitutively nuclear PER would fail to generate circadian rhythms, and tim^+ activity might play a role in temporal regulation of the access of PER to the nucleus. It was proposed that PER may directly or indirectly regulate its own transcription, because the cycling of *per* transcription is blocked in per^o mutants (*11*). Because the *tim* mutation also abolishes these RNA rhythms (*12*), a form of feedback regulation may exist in which cycling nuclear localization of PER might produce rhythmic signals influencing *per* transcription. We wish to emphasize that *tim* was recovered in a screen for clock mutations that was not biased to afford recovery of new mutations interacting with *per* (*12*). Thus, the discovery of a functional interaction between *tim* and *per* indicates that a single intracellular mechanism is probably central to the generation of circadian rhythms in *Drosophila*. The effects of *tim* on *per* RNA oscillation and PER nuclear localization would presumably be components of this mechanism.

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Activation of Phosphatidylinositol-3′ Kinase by Src-Family Kinase SH3 Binding to the p85 Subunit

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Engagement of antigen receptor complexes induces rapid activation of Src-family kinases and association with phosphatidylinositol-3′ kinase (PI-3 kinase). Here it was found that the Src homology 3 (SH3) domain of Lyn and Fyn bound to a proline-rich region (residues 84 to 99) within the 85-kilodalton subunit (p85) of PI-3 kinase. The binding of SH3 to the purified kinase led to a five- to sevenfold increase in the specific activity of PI-3 kinase. Ligand-induced receptor stimulation activated PI-3 kinase, and this activation was blocked by a peptide containing residues 84 to 99 of p85. These data demonstrate a mechanism for PI-3 kinase activation and show that binding of SH3 domains to proline-rich target sequences can regulate enzymatic activity.

The response of lymphocytes to antigen is mediated through a multisubunit cell surface receptor complex. Antigen receptor engagement can initiate multiple intracellular signaling events that lead to activation, differentiation, and tolerance induction, depending on the nature of the stimulus and differentiative stage of the cell (*1*). The binding of ligand to B cell antigen receptors causes activation of the receptor-associated Src-family tyrosine kinases $p55^{blk}$ (Blk), $p59^{fyn}$ (Fyn), and $p53/56^{lyn}$ (Lyn) (*2*). Lyn associates with the noncatalytic 85-kD subunit of PI-3 kinase in B cells after antigen receptor ligation (*3*).

The PI-3 kinase is a heterodimeric protein composed of a noncatalytic p85 subunit (*4*), catalytic 110-kD subunits (p110) (*5*), and phosphorylated inositol lipids on the D-3 hydroxyl position (*6*). The exact roles of the phosphoinositide products generated by PI-3 kinase in signaling pathways have not been determined, but they accumulate in cells activated by growth factors (*7*). PI-3 kinase interacts with SV40 middle

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T antigen–pp60$^{c\text{-}src}$ complexes (*8*) and stimulated growth factor receptors (*9–11*). Tyrosine-phosphorylated Tyr-X-X-Met motifs found in these molecules bind to SH2 domains of the p85 subunit (*8–13*) and activate PI-3 kinase (*14*). The PI-3 kinase binding site is essential for transduction of mitogenic signals by the platelet-derived growth factor receptor (*15*). Although a number of nonkinase receptors that activate Src-family kinases also activate PI-3 kinase, neither these receptors nor Src-family kinases contain Tyr-X-X-Met motifs. Therefore, these receptors must use a distinct mechanism for their association with and activation of PI-3 kinase.

The Src-family kinases contain two NH_2-terminal regions—the SH2 and SH3 domains—that potentially associate with intracellular effectors. Several Src-family kinases interact with PI-3 kinase (*3, 16*), and a single point mutation in the SH3 domain of Src (Lys^{106}→Glu) reduces PI-3 kinase binding (*17*). The catalytic and transforming potentials of p60$^{c\text{-}src}$ are modified by mutation of the SH3 domain (*18*). These findings suggest that interaction of pp60$^{v\text{-}src}$ with PI-3 kinase may mediate at least part of the kinase's transforming potential (*9, 19*). The mapping of the binding site for PI-3 kinase to the SH3 domains of several Src-family kinases (*20, 21*) is consistent with these mutational effects.

SH3 domains bind to proline-rich sequences. A consensus proline-rich sequence of 10 amino acids, XPXXPPPΨXP (where Ψ represents any hydrophobic residue and X represents any amino acid), is responsible for binding the protein 3BP-1 to the SH3 domain of Abl (*22*). Solution structure analysis reveals that SH3 domains contain a binding site for proline-rich sequences composed of well-conserved aromatic amino acids (*23*). Many of the amino acids in the binding site, however, show little conservation, suggesting that different SH3 domains may exhibit specificity for distinct proline-rich peptides (*23*).

We assessed the ability of peptides reflecting the two proline-rich regions within PI-3 kinase to inhibit binding of Lyn and Fyn to PI-3 kinase. These proline-rich sequences were KKISPPTPKPRPPRPTPVAPGSSKT (p85, residues 80 to 104) and NERQPAPATPPKPPKPTTVA (p85, residues 299 to 318) (*24*). The peptides Lyn (residues 27 to 131) (p56^{lyn}) and Fyn (residues 1 to 144), which contain the respective SH3 domains of the kinases, were coupled to Sepharose beads and used to probe detergent lysates of the K46 B cell lymphoma that contain PI-3 kinase (*20*) in the presence or absence of p85(80–104) and p85(299–318). We assessed the binding of the p85 subunit to Lyn and Fyn fusion proteins by immunoblotting proteins eluted from the beads. When present at twice the concentration of the fusion protein, p85(80–104) inhibited PI-3 kinase binding to Fyn by ~90% (as determined by scanning densitometry). The peptide completely blocked the association when it was present at concentrations 20 and 200 times that of the fusion protein. The p85(299–318) peptide inhibited PI-3 kinase association with Fyn when its concentration was at 200 times that of the fusion protein but showed no detectable inhibition of binding at lower concentrations (Fig. 1A). Similar results were observed with beads coupled to the SH3 of Lyn except that inhibition by both p85(80–104) and p85(299–318) was weaker (Fig. 1B), suggesting that Fyn may have a lower affinity for p85 than Lyn. Other proline-rich proteins have different affinities for distinct SH3 domains. The 3BP-1 protein binds to the SH3 domains of Abl, Src, Grb2, and Nck with decreasing affinities (*22*). These results are consistent with the possibility that the two proline-rich sites within the p85 subunit may also mediate PI-3 kinase binding to other SH3-containing proteins. These findings indicate that recognition of the sequence of residues 80 to 104 within p85 is necessary for SH3 binding to PI-3 kinase.

To determine the sufficiency of the proline-rich p85 sequences in mediating the association of PI-3 kinase with SH3 domains of Lyn and Fyn, we coupled p85(80–

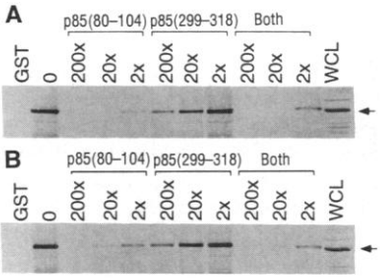

Fig. 1. Effects of p85(80–104) and p85(299–318) on association of p85 with Lyn and Fyn. Fyn(1–144) and Lyn(27–131) (p56^{lyn}) GST fusion proteins were cleaved and kinase fragments coupled to CNBr-activated Sepharose 4B beads (*20*). Clarified lysates [1% NP-40, 150 mM NaCl, 10 mM tris-HCl (pH 7.4), 2.0 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.4 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, aprotinin (2 mg/ml), leupeptin (2 mg/ml), and α-1-antitrypsin (2 mg/ml)] of K46 cells (1 × 10^7 cell equivalents per sample) were prepared and incubated with beads coated with either (**A**) Fyn(1–144) or (**B**) Lyn(27–131) (10 μl of a 50:50 slurry). Proline-rich peptides from p85 were added into mixtures in concentrations 200, 20, and 2 times that of Lyn and Fyn peptide on beads (*24*) and then incubated overnight. Directly coupled GST-peptide bead adsorptions were done as a control. The beads were then washed five times in lysis buffer, resuspended in sample buffer, and separated by SDS-PAGE (10%). Proteins were then transferred to Immobilon (Millipore) and probed with antibodies to p85 (Upstate Biotechnology, Inc.). Clarified lysate from solubilized cells (1 × 10^6 cell equivalents) was included as a control [whole cell lysate (WCL)]. Membranes were thoroughly washed in tris-buffered saline (TBS) containing 0.02% Triton X-100 and subsequently incubated with goat antibody to rabbit IgG conjugated with alkaline phosphatase (Fisher). The membranes were washed again and developed with alkaline phosphatase substrate (Vector Labs, Inc.). Data are representative of three independent experiments. The position of p85 is indicated by an arrow.

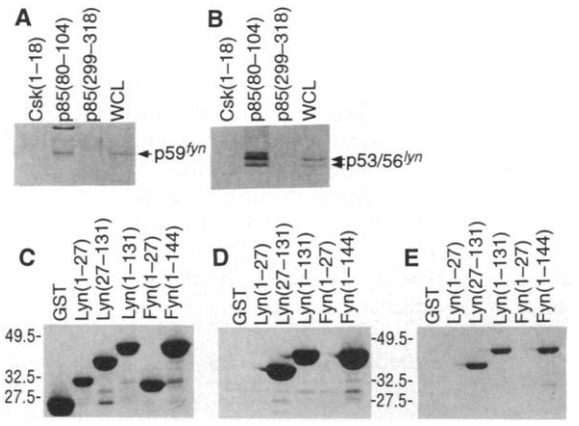

Fig. 2. Association of immobilized proline-rich regions of p85 with SH3 domains of Lyn and Fyn. Proteins from K46 lysates (1 × 10^7 cell equivalents per sample) were adsorbed to beads coupled to p85(80–104), p85(299–318), or an irrelevant peptide [Csk(1–18)]. Eluates were fractionated and then immunoblotted with antibodies to Fyn (**A**) or Lyn (**B**). Whole cell lysate (WCL) (1 × 10^6 cell equivalents) was included as a control. Adsorption, fractionation, and immunoblotting were performed essentially as described in Fig. 1. Position of Fyn (A) and Lyn (B) are indicated to the right with an arrow. Bacterial lysates containing GST fusion proteins [Lyn(1–27), Lyn(27–131), Lyn(1–131), Fyn(1–27), and Fyn(1–144)] were adsorbed with (10 μl of a 50:50 slurry) glutathione-Sepharose beads (Pharmacia) (**C**), p85(80–104)–coupled beads (**D**), or p85(299–318)–coupled beads (**E**). (Peptides were coupled to CNBr-activated Sepharose 4B beads at a ratio of 2 mg of peptide per milliliter of activated beads according to instructions provided by manufacturer.) Adsorbates were washed thoroughly with lysis buffer, resuspended in sample buffer, and fractionated by SDS-PAGE (10%). Proteins were visualized by Coomassie blue staining. *Escherichia coli* DH5α cells expressing respective GST fusion proteins were collected from 2-ml cultures, and lysates prepared as described (*20*).

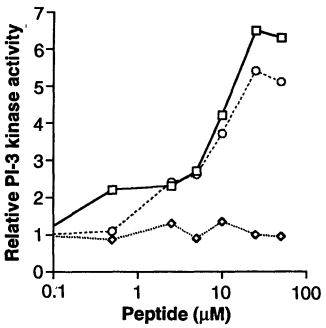

Fig. 3. Augmentation of PI-3 kinase enzymatic activity by peptides containing SH3 domains of Lyn and Fyn. Antibodies to the p85α subunit (1 μl of whole serum, Upstate Biotechnology, Inc.) were added to clarified lysates of K46 cells (1 × 10^7 per sample). Immune complexes were then precipitated with 40 μl of (50:50 slurry) protein A beads (Pharmacia). Adsorbates were washed and divided into equal portions and added to lysis buffer containing various concentrations of Lyn(27–131) (□), Fyn(1–144) (○), or Blk(1–108) (◇), peptides that were prepared by cleavage of GST fusion proteins with Factor Xa (*20*). After incubation overnight at 4°C, adsorbates were thoroughly washed in PAN buffer [100 mM NaCl, 20 mM Pipes (pH 7), and aprotinin (2 mg/ml)]. PI-3 kinase was then assayed as described (*20*). Amount of activity is expressed relative to that of cells not exposed to peptide. Plates were dried and phospholipids quantitated by PhospholmagerTM with ImageQuant software. Results are representative of four experiments.

104) and p85(299–318) to Sepharose 4B beads and used them to probe lysates of K46 cells. Adsorbed proteins were eluted by boiling in sample buffer, subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nylon membranes, and immunoblotted for Fyn (Fig. 2A) and Lyn (Fig. 2B). Binding of p85(80–104) to Lyn and Fyn was easily detectable. The p85(299–318) peptide exhibited no detectable binding activity. Immunoreactive Lyn or Fyn did not bind to a control peptide containing residues 1 to 18 of the mouse Csk protein. Thus, the proline-rich region spanning residues 80 to 104 of PI-3 kinase contains sufficient information to mediate Src-family kinase SH3 binding to p85; however, formal in vivo mapping remains to be done.

To determine if binding of p85(80–104) to SH3 domains of Lyn and Fyn is a direct interaction, we probed detergent lysates of bacteria expressing truncated Lyn– and Fyn–glutathione-S-transferase (GST) fusion proteins (*20*) with beads coupled to p85 proline-rich peptides. As determined by Coomassie staining of gels after SDS-PAGE, the p85(80–104) peptide bound only the Lyn and Fyn fusion proteins that contained SH3 domains [Lyn(27–131), Lyn(1–131), and Fyn(1–144)] (Fig. 2D). The p85(299–318) peptide bound the same proteins as p85(80–104), albeit with much lower affinity (Fig. 2E). Adsorption to glutathione-Sepharose beads established that each bacterial lysate contained equivalent amounts of GST fusion proteins (Fig. 2C). Control adsorptions with glycine-Sepharose beads confirmed that nonspecific binding to Sepharose beads did not occur. These data indicate that the proline-rich regions within the p85 subunit of PI-3 kinase can mediate selective and direct association with the SH3 domains of Lyn and Fyn.

We next assessed the effect of SH3 binding on PI-3 kinase activity. SH3-containing polypeptides derived from both Lyn and Fyn increased the kinase activity of isolated PI-3 kinase by approximately seven- and fivefold, respectively, whereas Blk, which does not associate with PI-3 kinase (*20*), had no effect (Fig. 3). This activation was half-maximal in the presence of ~10 μM peptide and was blocked by the addition of 100 μM p85(80–104) (*25*). In control experiments, fragments of the unique region of Lyn and Fyn failed to stimulate PI-3 kinase activity (*25*). These data indicate that SH3 domains of Lyn and Fyn can directly stimulate PI-3 kinase activity, presumably by binding to proline-rich regions within the p85 subunit. This activation is independent of both p85 subunit phosphorylation and binding of phosphotyrosine-containing peptide to p85. The data suggest that the increase in precipitability of p85 with antibodies to phosphotyrosine that occurs after antigen receptor stimulation (*26*) may reflect increased association of PI-3 kinase with phosphorylated Src-family kinases or CD19 or both (*27*) rather than phosphorylation of PI-3 kinase itself.

We then assessed the importance of the SH3-p85(80–104) interaction in antigen receptor–mediated PI-3 kinase activation. PI-3 kinase was immunoprecipitated from purified resting B cells that were unstimulated or stimulated with antibody to B cell antigen receptor and its activity assessed. Antigen receptor ligation led to an approximately sevenfold increase in PI-3 kinase activity that was maximal at 1 min (Fig. 4). Normal B cells were permeablized to allow entrance of p85(80–104) and p85(299–318) and then stimulated through their antigen receptors for 1 min with antibodies to immunoglobulin M and D (anti-IgM and anti-IgD) (*28*). The cells were lysed, and PI-3 kinase was immunoprecipitated and assayed for activity. In the presence of p85(80–104), stimulation of PI-3 kinase activity by anti-Ig was completely blocked (Fig. 4). The p85(299–318) peptide had no detectable effect on receptor-mediated activation of PI-3 kinase activity. Essentially identical results were obtained when the B cell lymphoma line K46μm17 was used (*25*). Immunoblotting with anti-p85 antisera (see Fig. 1) confirmed that equivalent amounts of p85 were present in each precipitate (*25*). Probing of duplicate blots with antibodies to phosphotyrosine revealed no detectable phosphorylation of p85 (*25*). This suggests that the observed increase in PI-3 kinase activity that follows stimulation of a B cell surface receptor complex is independent of tyrosine phosphorylation of p85. Activation of PI-3 kinase induced by antibody to CD3 is also independent of tyrosine phosphorylation (*29*). These findings suggest that PI-3 kinase activation occurs through direct binding of the SH3 domain of a Src-family kinase after T and B cell antigen receptor ligation but do not formally exclude the role of another p85(80-104) binding protein in this response.

Several mechanisms for activation of PI-3 kinase have been proposed. The PI-3 kinase p85 subunit has been shown to be tyrosine-phosphorylated in cells transformed with middle T antigen and after stimulation of the growth factor receptor (*13*). This phosphorylation correlates with increased enzymatic activity and causality has been suggested (*30*). Tyrosine-phosphorylated Tyr-X-X-Met peptide motifs activate PI-3 kinase by associating with SH2 domains of p85 (*14*). Our data demonstrate another mode of PI-3 kinase

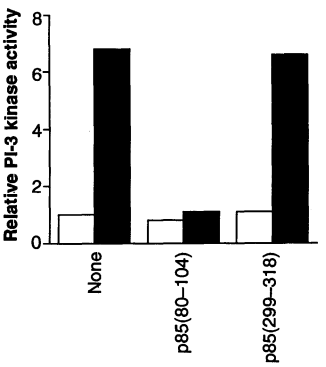

Fig. 4. Inhibition by p85(80–104) of antigen receptor–mediated PI-3 kinase activation. Resting mouse B cells (ρ > 1.062) were isolated by Percoll density gradient sedimentation (*28*). Cells (5 × 10^7 cells per sample) were permeabilized in Mire's buffer [10 mM $MnCl_2$, 2 mM EGTA, and 296 μM $CaCl_2$ (Ca^{2+} buffered to 30 nM), 1 mM 2-mercaptoethanol, 40 mM Hepes (pH 7.4), α-lysophosphatidylcholine (285 μg/ml), and palmitoyl] (*28*) either alone or containing 1.4 mM p85(80–104) or p85(299–318) on ice for 1 min. Cells were warmed for 2 min at 37°C before incubation for 5 min at 37°C in the presence of either phosphate-buffered saline alone (open bars) or with monoclonal anti-IgD (JA12) and anti-IgM (b-7-6) (50 μg/ml of each) (solid bars). Cells were then collected by centrifugation, lysed in 1% NP-40 containing buffer (0.5 ml), and incubated for 30 min on ice. Nuclei were removed by centrifugation (14,000*g* for 15 min). To each sample was added 50 μl (50:50 slurry) of protein A–Sepharose (Pharmacia) and 4 μl of antiserum to p85 (Upstate Biochemicals, Inc.) before overnight incubation at 4°C. Adsorbates were washed five times with 1% NP-40 lysis buffer and twice with PAN buffer. Portions (5 μl of a 50:50 slurry) of each precipitate were then removed and assayed for PI-3 kinase enzymatic activity (Fig. 3). Results are representative of four independent experiments.

regulation wherein binding of Src-family kinase SH3 domains to p85 increased enzymatic activity and indicate that this mechanism is operative during antigen receptor signaling.

Cross-linking of CD19 and membrane IgM (mIgM) on B cells induces tyrosine phosphorylation of Tyr-X-X-Met motifs on CD19 and association of CD19 with PI-3 kinase (*27*). Thus, PI-3 kinase activation through Src-family kinase SH3 domains may be amplified by the binding of tyrosine-phosphorylated CD19 to p85 SH2 domains. Each of these mechanisms could localize PI-3 kinase to the membrane, which may be important for enzyme access to substrate (*31*).

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Requirement for Transcription Factor IRF-1 in NO Synthase Induction in Macrophages

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Production of nitric oxide (NO) by macrophages is important for the killing of intracellular infectious agents. Interferon (IFN)-γ and lipopolysaccharide stimulate NO production by transcriptionally up-regulating the inducible NO synthase (iNOS). Macrophages from mice with a targeted disruption of the IFN regulatory factor–1 (IRF-1) gene (IRF-1$^{-/-}$ mice) produced little or no NO and synthesized barely detectable iNOS messenger RNA in response to stimulation. Two adjacent IRF-1 response elements were identified in the iNOS promoter. Infection with *Mycobacterium bovis* (BCG) was more severe in IRF-1$^{-/-}$ mice than in wild-type mice. Thus, IRF-1 is essential for iNOS activation in murine macrophages.

Nitric oxide is an unstable free radical gas that functions as an intercellular messenger in vasodilation and neurotransmission and as a cytotoxic agent of macrophages (*1*). Three related genes encode NO synthases (NOS) in different tissues. Endothelial and neuronal NOS are constitutive enzymes whose functions are activated by Ca^{2+}-calmodulin. Macrophages and some other cells have a transcriptionally inducible form of NOS (iNOS) that remains undetectable until these cells are activated. Interferon-γ and bacterial lipopolysaccharide (LPS) are the most potent activators of the iNOS gene in murine macrophages (*2*). Induced NO production is one of the principal mechanisms of macrophage cytotoxity for tumor cells, bacteria, protozoa, helminths, and fungi (*3*); NO also participates in antiviral defenses (*4*). The promoter region of the murine iNOS gene is responsive to stimulation by IFN-γ and LPS on transfection into a murine macrophage cell line (*5*, *6*).

Interferon regulatory factor–1 (IRF-1) and the related IRF-2 protein bind to sites within the promoters of IFN-α, IFN-β, and several IFN-inducible genes (*7*–*12*). IRF-1 activates transcription whereas IRF-2 inhibits it (*8*, *9*). The IRF-1 gene is induced by treatment with IFNs, and also by tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, and leukemia inhibitory factor (*13*). Mice were generated with homozygous targeted disruptions of the IRF-1 or IRF-2 genes (*14*). IRF-1$^{-/-}$ mice show no overt abnormalities either during embryonic development or postnatally. The major defect found in IRF-1$^{-/-}$ mice was a 90% reduction in the number of CD8$^+$ T cells. Although embryonic fibroblasts from IRF-

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